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1. **Holland et al. Anal. Biochem. 1994, 222(2), pp. 516-518.**
2. **Peterson et al. Anal. Biochem. 1999 (7/1/99), 271(2), pp. 131-136.**
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4. **Park et al. Anal. Biochem. 1999 (4/10/99), 269(1), pp. 94-104.**

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# ANALYTICAL BIOCHEMISTRY

## Methods in the Biological Sciences

*Review:*

**Immunotargeting Intracellular Compartments**

*Vic Raso*

ACADEMIC PRESS, INC.
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## A Nonseparation Microplate Receptor Binding Assay

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Screens for novel receptor agonists or antagonists based on conventional vacuum filtration technology are cumbersome and time-consuming when run at high throughput. Ideally, a nonseparation assay is required to minimize the manipulations involved. The principle of scintillation proximity counting using beads containing scintillant is one such approach to nonseparation assays (1–4). In the most often used configuration (2–4), a protein "receptor" component is coated onto the beads and incubated with the radiolabeled ligand; bound counts are sufficiently close to the beads to excite the scintillant, while the more distant unbound counts are not detected.

We have evaluated a new microtiter plate coated with scintillant as a means of exploiting this principle in a convenient format. The FlashPlate (Packard) is an opaque, 96-well polystyrene microtiter plate with a plastic scintillant coating sensitive to, inter alia, $^{125}I$ Auger electrons. The coating was selected to provide a similar surface to polystyrene to allow attachment of proteins of interest by passive coating. We found that cell membrane fragments also can be passively coated to this surface, suggesting a convenient nonseparation receptor binding assay. To investigate this possibility, we selected the human endothelin $ET_A$ receptor as a model.

*Materials and methods.* Receptor membrane preparations were obtained from MEL cells (5) engineered to express recombinant human endothelin-A receptor (6) and were kindly provided by C. M. Shaw (ZENECA). Cells were homogenized, using a Polytron homogenizer, in 50 mM Tris·HCl, pH 7.4, containing soybean trypsin inhibitor (5 μg/ml), 1,10-phenanthroline (1 mM), benzamidine (1 mM), bacitracin (100 μg/ml), and sucrose (3 mM), and centrifuged at 1500*g* for 10 min at 4°C. Supernatants were recentrifuged at 40,000*g* for 30 min and the pellet was washed once and then resuspended in the above buffer using a glass/Teflon hand-held homogenizer.

Human endothelin-1 was obtained from Cambridge Research Biochemicals. [$^{125}I$]Endothelin-1 (human, 2200 Ci/mmol, NEX-259) was obtained from NEN. Angiotensin II was obtained from Bachem. Other peptides were obtained from Sigma. XT401 and XT402 are endothelin antagonists under investigation in ZENECA Pharmaceuticals and were kindly provided by Anand Dutta and Roger James.

FlashPlates (Packard Instrument Co.) were coated with receptors (0.5 mg/ml, 100 μl) and blocked with BSA[2] as described by Nichols *et al.* (7). In brief, membranes (0.05 mg/ml total protein) were added to each well in 0.15 M $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.5 (100 μl). The plates were then centrifuged at 800*g* for 10 min at 4°C and the supernatants were removed. Blocking was achieved with BSA (5 mg/ml, 200 μl) for 30 min.

To the coated plates were added unlabeled endothelin or test compound (30 μl) and [$^{125}I$]endothelin (30 pM final) in 50 mM Tris·HCl, 1 mM $CaCl_2$, 0.1% BSA, 0.05% Tween 20, pH 7.4, in a total volume of 90 μl. Controls comprised (a) totals, radioligand only, and (b) nonspecific binding, 0.1 μM (final) unlabeled endothelin. Reference controls with 0.3 nM unlabeled endothelin were also employed in some experiments; this gives ca. 50% displacement. The plates were then sealed with TopSeal-S using a Packard Micromate 496, incubated at room temperature for 16 h, and counted on a Packard Top Count microplate scintillation and luminescence counter. A 1-min counting period was used. All determinations were carried out at least in triplicate.

As a comparison, compounds were also tested in a filtration-based assay as follows. Receptors (0.15 mg/ml protein, 30 μl) and [$^{125}I$]endothelin (90 pM, 30 μl) were incubated with cold endothelin or test compound (30 μl) in 50 mM Tris·HCl, 1 mM $CaCl_2$, 0.1% BSA, 0.05% Tween 20, pH 7.4, at ambient temperature for 2.5 h. The suspensions were then filtered through a prewetted glass fiber filter (printed filter mat B, Wallac) on a 96-well harvester (Tomtec 96) and washed with 50 mM Tris·HCl, pH 7.4. The filters were then either dried and loaded into sample bags with scintillant (Beta-Scint, Wallac), and counted on a Wallac 1205 Betaplate counter, or cut into sections and counted on a gamma counter (Wallac 1277).

*Results.* We found that of the total counts added in the nonseparation assay, ca. 10,000 cpm in this study,

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[2] Abbreviation used: BSA, bovine serum albumin.

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TABLE 1
Comparison of Assay Characteristics

| | Filtration assay (SD, $n$ = 10) | FlashPlate assay (SD, $n$ = 8) |
|---|---|---|
| Counts added | 11,290 (122) | 9707 |
| Total binding | 1792 (48) | 1591 (94) |
| Nonspecific binding | 280 (16) | 151 (19) |
| 50% control | 895 (22) | 723 (69) |
| Specific/total binding | 83% | 90% |
| Fraction total counts bound | 15.9% | 16.4% |

TABLE 2
Activity of Compounds

| Compound | $IC_{50}$ (nM) | |
|---|---|---|
| | Filtration assay | FlashPlate assay |
| Endothelin-1 | 0.21 | 0.13 |
| Endothelin-2 | 0.24 | 0.18 |
| Endothelin-3 | 29 | 70 |
| XT401 | 426 | 263 |
| XT402 | 3.2 | 9.8 |
| Sarafotoxin 6c | 2690 | >100 |
| Angiotensin II | >10,000 | >10,000 |

ca. 0.9% were detected in untreated wells. This figure rises to 16% in wells coated with receptor (see below). This clearly illustrates the ability of the plates to detect bound counts in the presence of unbound counts.

Key assay data for the nonseparation and conventional assays are compared in Table 1. Loadings for the FlashPlate assay were designed to give similar counts to the vacuum filtration assay. This was achieved with similar levels of receptor (4.5 and 5.0 $\mu$g protein per well, respectively) and the percentage of total counts bound was similar in both cases (ca. 16%). The signal-to-background ratio was slightly better for the nonseparation assay: 90% of the bound counts were specific, compared to 83% for the filtration assay. The reproducibility of both assays is similar. It should be noted that a significant component of the nonspecific counts is due to the "proximity" background due to unbound counts being within the detection volume of the scintillant; this is an inherent, though consistent, feature of the assay. This component, 0.9% of added counts, corresponds to ca. 5.6% of total bound counts and ca. 60% of the nonspecific counts.

In this approach, the binding of the radiolabel may be followed in real time; this is a useful feature in assay development. The time course (not shown) indicates that in this case overnight incubation is appropriate, though the majority of the binding takes place within 5 h. The slower binding may be related to the greater diffusion distances that must be overcome with the receptor immobilized on the plate, compared to in a suspension in the conventional assay. However, preliminary investigations with other receptors show much faster kinetics than those obtained with the endothelin receptor (P. Singh and M. Walters, unpublished observations), reaching equilibrium in a few hours, despite an apparently identical diffusion barrier. This aspect requires further investigation. Overnight incubation is not a significant disadvantage for high-throughput screening.

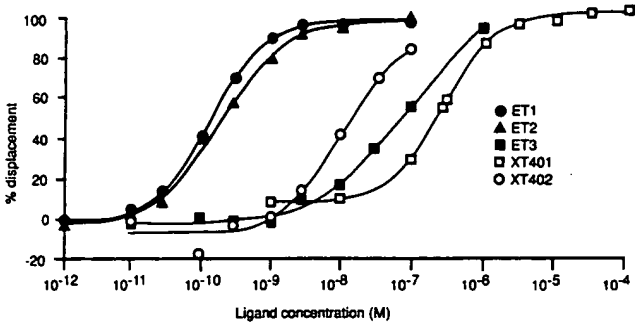


FIG. 1. Displacement of endothelins and endothelin antagonists in the FlashPlate assay.

Displacement curves for unlabeled endothelin and endothelin antagonists are shown in Fig. 1. Displacement curves are parallel and the relative potency of the endothelins and of sarafatoxin 6c is ET-1 $\geq$ ET-2 > ET-3 $\gg$ sarafatoxin 6c. This is consistent with that reported for a classical $ET_A$ receptor (8,9). The $IC_{50}$ values obtained in both assays are shown in Table 2. The agreement between the two methods is within the range found typically when several determinations of $IC_{50}$ are performed using vacuum filtration and is at least adequate for high-throughput screening purposes.

*Discussion.* The key feature of the coated FlashPlate assay described here is its simplicity; in essence, it comprises the addition of the radioligand and test compound, incubation, and measurement. No separation or washing steps are involved. In a high-throughput screening context, this simplicity makes the assay more amenable to automation. Also, there are fewer components and steps which can fail; this allows for a more robust assay.

We are currently attempting to devise simplified plate-coating procedures and have shown that omission of the centrifugation step, though giving reduced ligand binding, nevertheless gives a satisfactory signal for highly expressed receptors (unpublished data).

Although designed for a relatively crude primary screen for compounds interacting at a receptor, the precision of the assay and its correlation with the conventional approach suggest that it is suitable for a vari-

ety of quantitative receptor binding studies. Unlike separation-based assays, this assay does not disturb the equilibrium, making it suitable for measuring weaker ligand–receptor interactions. In summary, we conclude that receptor-coated FlashPlates provide a powerful new approach to the study of receptor interactions and the identification of new receptor binding entities of pharmaceutical or agrochemical relevance.

*Acknowledgments.* We thank C. M. Shaw and the staff of the ZENECA Pharmaceuticals Tissue Culture Unit for the supply of cells for the membrane preparations, Elaine Whiting and Karen Taylor for technical support, Eric Tang for modifications to the membrane preparation methodology, and John Major and Mark Beggs for useful discussions. We are also grateful to Packard Instrument Co. for the prelaunch supply of the FlashPlates. The MEL cell system and vectors are covered by issued and pending patent rights held by the Medical Research Council and ZENECA, to whom all inquiries should be addressed.

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